Multiple binding sites involved in the effect of choline esters on decarbamylation of monomethylcarbamoyl- or dimethylcarbamoyl-acetylcholinesterase

Dai-Eun SOK,* Yun-Bae KIM, Seung-Ju CHOI, Chang-Hee JUNG and Seung-Hee CHA

Advanced Technology Research Center (no. 6–7), Agency for Defense Development, P.O. Box 35, Yuseong, Taejeon 305-600, Korea

Multiple binding sites for inhibitory choline esters in spontaneous decarbamylation of dimethylcarbamoyl-acetylcholinesterase (AChE) were suggested from a wide range of IC_{50} values, in contrast with a limited range of AC_{50} values (concentration giving 50% of maximal activation) at a peripheral activatory site. Association of choline esters containing a long acyl chain (C_7 – C_{12}) with the hydrophobic zone in the active site could be deduced from a linear relationship between the size of the acyl group and the inhibitory potency in either spontaneous decarbamylation or acetylthiocholine hydrolysis. Direct support for laurylcholine binding to the active site might come from the competitive inhibition (K_i 33 μ M) of choline-catalysed decarbamylation by laurylcholine. Moreover, its inhibitory action was greater for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE, where there is a greater steric hindrance at the active centre. In further support, the inhibition of

pentanoylthiocholine-induced decarbamylation by laurylcholine was suggested to be due to laurylcholine binding to a central site rather than a peripheral site, similar to the inhibition of spontaneous decarbamylation by laurylcholine. Supportive data for acetylcholine binding to the active site are provided by the results that acetylcholine is a competitive inhibitor (K_i 7.6 mM) of choline-catalysed decarbamylation, and its inhibitory action was greater for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE. Meanwhile, choline esters with an acyl group of an intermediate size (C_4 – C_6), more subject to steric exclusion at the active centre, and less associable with the hydrophobic zone, appear to bind preferentially to a peripheral activity site. Thus the multiple effects of choline esters may be governed by hydrophobicity and/or a steric effect exerted by the acyl moiety at the binding sites.

INTRODUCTION

It was reported previously [1] that choline potentiated spontaneous decarbamylation of methylcarbamoyl-acetylcholinesterase (AChE) by interacting with the central active site of the modified enzyme. Subsequently, the choline-catalysed decarbamylation was observed to be affected by the allosteric effectors such as gallamine and tubocurarine, which are known to interact with the peripheral anionic site of the enzyme [2–4]. Additionally decamethonium, which was reported to occupy simultaneously both a central and a peripheral anionic site, was observed to enhance dephosphorylation of alkylphosphorylated enzyme [5]. Thus, spontaneous decarbamylation or dephosphorylation seems to be positively regulated via two distinct binding sites.

Recently [6] it was reported that choline esters or thiocholine esters with acyl chain of intermediate size (C_4 – C_6) enhanced choline-catalysed decarbamylation of dimethylcarbamoyl-AChE. The activatory action of those choline esters was shown kinetically to be peripheral, compared with the central action of choline, with a K_a value of 1.4 mM, and the binding affinity of those choline esters at the activatory peripheral site was observed to be as low as the binding affinity of the substrate. Moreover, the activation by choline esters exhibited a strict structural requirement and no dependence on ionic strength. On the other hand, choline esters containing long acyl groups or excess acetylcholine were found to show an inhibitory influence on decarbamylation. However, it was not clarified whether the inhibitory action of these compounds occurred on a peripheral

site or a central one. Although the substrate inhibition had been proposed to be due to the binding of acetylcholine to the active centre of AChE [1,7], more recent studies suggest that the binding site for substrate inhibition may be a peripheral anionic site [8,9].

There exist two classes of inhibitors of AChE activity; one class covers the structurally diverse cationic organic compounds interacting with the active centre [1,10,11] and the other includes ligands associable with a peripheral site remote from the active centre [12–15]. As a result of extensive kinetic studies, these multiple inhibitors have been characterized by competitive, non-competitive and uncompetitive patterns of inhibition.

Although the active site of AChE has been studied by multidisciplinary approaches, this resulted in intricate theories explaining catalytic properties and inhibitor interaction. Recently, several separate amino acid domains were suggested to be involved in the active site of the enzyme [16], and subsequent three-dimensional analysis of *Torpedo* AChE clarified the understanding of the active site [17]. Interestingly, the acetylcholine-binding site was found to be hydrophobic rather than anionic, since 14 aromatic amino acid residues were observed to line the surface of the cleft in the active site [17]. Different inhibitor sensitivity was supposed to be due to differences in charge and/or hydrophobicity at the rim domain, within the active-site cleft and in structurally important regions [16]. Therefore a comprehensive study using several ligands with similar, but not identical, properties was suggested to be valuable for the classification of various binding subsites. In this regard, the binding sites responsible for the inhibitory action of choline

Abbreviations used: AChE, acetylcholinesterase; AC_{50} , concentration giving 50% of maximal activation.

* To whom correspondence should be addressed.

esters with acyl moieties of various sizes in decarbamylation of monomethylcarbamoyl- and dimethylcarbamoyl-AChE were examined kinetically.

Here, we demonstrate that the binding sites for choline esters may be determined by the steric effect and the hydrophobicity exerted by the particular choline ester at the binding site, and we propose that laurylcholine or acetylcholine inhibits spontaneous or choline-catalysed decarbamylation of alkylcarbamoyl-AChE by binding primarily to the active site of the modified enzyme.

EXPERIMENTAL

Materials

Pyridostigmine bromide, physostigmine salicylate and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co., and choline chloride was provided by Merck. Various choline esters or thiocholine esters were also from Sigma, except for the choline esters such as pentanoylcholine iodide, hexanoylcholine iodide, heptanoylcholine iodide, octanoylcholine iodide and decanoylcholine iodide, synthesized as described previously [18], and the thiocholine esters such as pentanoylthiocholine iodide, hexanoylcholine iodide, heptanoylcholine iodide, octanoylcholine iodide and laurylthiocholine iodide, prepared as described previously [6].

Assay of AChE

This was performed at 25 °C by a slight modification [6] of the method of Ellman [19]. The assay mixture (3.0 ml) contained 1.4 mM acetylthiocholine, 1.0 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 100 mM NaCl and 100 mM sodium phosphate, pH 7.4. Turbidity of the enzyme homogenate was confirmed to have negligible effect on both the enzyme activity and the decarbamylation rate under this assay condition. Corrections were made for the non-specific hydrolysis of substrate, including non-enzymic hydrolysis.

Preparation of monomethylcarbamoyl- or dimethylcarbamoyl-AChE

Whole mouse brain was washed and homogenized in 15 vol. of the incubation buffer, consisting of 0.2 mM CaCl₂, 1.0 mM MgCl₂ and 50 mM sodium phosphate, pH 7.35. The homogenate was centrifuged at 600 *g* for 10 min to remove crude tissues and blood contamination. Subsequently the supernatant was centrifuged at 25000 *g* for 10 min at 4 °C, and the pellet was rehomogenized in the above incubation buffer to obtain 10% (w/v) membrane-bound enzyme homogenate. This enzyme homogenate (enzyme activity approx. 0.6 µmol/min per ml) was incubated with pyridostigmine (final concn. 3 µM) or physostigmine (final concn. 5 µM) for 40 min at 37 °C with gentle shaking, resulting in over 90% inhibition of the enzyme activity, and then washed with 3 × 15 vol. of NaCl or 2 mM sodium phosphate buffer, pH 7.35 to remove the residual carbamate molecules. Finally, the pellet was rehomogenized in water before use to make 10% homogenate of the inhibited enzyme, divided into batches and stored at 4 °C without significant change in extent of carbamylation up to 24 h.

Decarbamylation of the monomethylcarbamoyl- or dimethylcarbamoyl-AChE

The decarbamylation reaction was started by adding a portion (10 µl) of the inhibited enzyme to the pre-warmed (37 °C) incubation buffer (500 µl) containing test compounds, and main-

tained at 37 °C under shaking. At each time interval, a 25 µl portion was taken and subjected to the AChE assay. The degree of decarbamylation was expressed as a percentage of the activity re-activated during each incubation period out of the total amount of the initially inhibited enzyme, as described previously [6,20]. The first-order rate constants (k'_r , the first-order rate constant for spontaneous decarbamylation; k_r , observed first-order rate constant in the presence of each test compound) were derived from the semi-logarithmic plots of the percentage inhibition [21] at three (5, 10 and 15 min) or two (5 and 10 min in the incubation containing acetylcholine) incubation time intervals. These experimental conditions were designed to minimize the effect of choline produced from choline esters during decarbamylation, and to allow the concentration of thiocholine produced from 1.5 mM acetylthiocholine during incubation to be lower than 10 µM. In a separate experiment where Triton X-100 (0.3%) was included in the decarbamylation mixture containing choline and/or some choline esters, to monitor the effect of turbidity, no significant difference in decarbamylation was observed.

Inhibition of acetylthiocholine hydrolysis and decarbamylation

For the inhibition of AChE activity, the appropriate choline ester was added to the assay mixture, and the inhibition constants, a competitive (K_{iB}) and a non-competitive (K_{iES}) component, were determined from double-reciprocal plots of reaction velocity against substrate concentration [1]. Also, the initial-velocity (v) measurements were analysed by non-linear regression. For the inhibition of choline-catalysed decarbamylation, K_i values were calculated directly from v measurements, which were analysed by a non-linear regression:

$$v(k_r - k'_r) = \frac{V_m [S]}{K_m} \left(1 + \frac{[S]}{K_m} + \frac{[I]}{K_i} \right)$$

where [S] represents the concentration of choline, and K_i is a competitive inhibition constant in choline-catalysed decarbamylation. The non-linear regression analyses were performed by the non-linear least-squares curve-fitting routine supplied with the data and analysis software of SigmaPlot 5.0 (Jandel Corp., U.S.A.). Representative double-reciprocal plots are presented for illustrative purposes. Separately, the inhibition of spontaneous decarbamylation was expressed as the percentage (%) of total spontaneously recovered activity, and the IC₅₀ value is the concentration for 50% inhibition of spontaneous decarbamylation. Inhibition of pentanoylthiocholine-induced decarbamylation was performed as described in the Figure legends.

Effect of pH and ionic strength on decarbamylation

Decarbamylation was carried out in the presence of choline and/or choline esters in the incubation buffer adjusted to various pH values (6.0–8.0), and the half-life ($t_{1/2}$) calculated from the first-order rate constant (k_r) was plotted against pH [21]. In a separate experiment to obtain the p*K*_a value for decarbamylation, the net first-order rate constant [$k_r - k'_r$; k_r , the first-order rate constant for choline (1 mM)-catalysed decarbamylation; k'_r , the first-order rate constant for spontaneous decarbamylation] was plotted against various pH values (6.0–9.0). The p*K*_a value was determined by obtaining the pH value for 50% of the maximal decarbamylation rate. The influence of ionic strength on the decarbamylation was investigated by using 2 mM phosphate buffer, pH 7.35, and the same buffer containing 150 mM NaCl, and the decarbamylation rate was estimated as described above.

RESULTS

Effect of choline esters on spontaneous decarbamoylation

Previously, it had been reported that various quaternary or tertiary ammonium compounds inhibited the spontaneous decarbamoylation of monomethylcarbamoyl-AChE [1]. In this study, the inhibitory effect of choline esters or thiocholine esters on the decarbamoylation of dimethylcarbamoyl-AChE was examined, by using compounds with acyl chains of various sizes. As shown in Figure 1(a), laurylcholine decreased spontaneous decarbamoylation of dimethylcarbamoyl-AChE in a concentration-dependent pattern (0.05–2 mM) which appears to consist of two components; a higher-affinity phase (0.05–0.2 mM), with an IC_{50} value of 170 μ M, and a lower-affinity phase (0.2–2 mM). The relative affinity between the two types was estimated to be approx. 5-fold different. However, choline esters with an acyl group of intermediate size, such as butyrylcholine, pentanoylthiocholine or hexanoylthiocholine, exhibited a concentration-dependent activation, which reached a maximal constant level throughout the 0.2–1 mM range (Figure 1b). Significant inhibition by these choline esters (C_4 – C_6) below the level of spontaneous decarbamoylation was accomplished by concen-

Figure 1 Effect of the choline ester or thiocholine ester on spontaneous decarbamoylation of dimethylcarbamoyl-AChE

Choline esters or thiocholine esters at various concentrations were incubated with dimethylcarbamoyl-AChE from mouse brain for 5 min in 50 mM sodium phosphate buffer, pH 7.35 (incubation buffer), at 37 °C. A portion (25 μ l) was used for the assay of enzyme activity as described in the Experimental section. Degree of decarbamoylation was denoted as the percentage of the fully decarbamoylated enzyme activity. The results are expressed as averages of three experiments. (a) Laurylcholine (○); (b) butyrylcholine (○), pentanoylthiocholine (●), hexanoylthiocholine (△); (c) acetylcholine (○), acetyl-(β -methyl)choline (●). Spontaneous decarbamoylation was denoted separately (----).

Figure 2 Lineweaver-Burk plot for the inhibition of choline-catalysed decarbamoylation by laurylcholine or acetylcholine

Choline-catalysed decarbamoylation of dimethylcarbamoyl-AChE was determined in the presence of laurylcholine (a) at various concentrations (0–100 μ M) or acetylcholine (b) of various concentrations (0–20 mM) as described in the Experimental section, where: $k_t = k'_t$ = net first-order rate constant; k_t = observed first-order rate constant for choline-catalysed decarbamoylation in the presence of laurylcholine (0–100 μ M) or acetylcholine (0–20 mM); k'_t = observed first-order rate constant for spontaneous decarbamoylation in the presence of laurylcholine (0–100 μ M) or acetylcholine (0–20 mM). The first-order rate constants were determined from the semi-log plots of degree of inhibition (%) at three incubation time intervals (5, 10 and 15 min). The rate constant for spontaneous decarbamoylation in the absence of inhibitor ranged between 0.0013 and 0.0015 min^{-1} . Kinetic analysis was performed as described in the Experimental section.

trations far above 5 mM. Meanwhile, the decarbamoylation by acetylcholine followed a dose-dependent increase below 0.2 mM, but decreased gradually beyond 0.5 mM, indicative of a biphasic effect (Figure 1c). The similar biphasic pattern was also observed with acetyl-(β -methyl)choline. Although acetylcholine, showing an AC_{50} value (concentration for 50 % of maximal activation) of approx. 50 μ M appeared to be as effective as pentanoylthiocholine (AC_{50} approx. 70 μ M) as an activator, its inhibitory action (IC_{50} approx. 10 mM) was much smaller than that of laurylcholine. Thus the binding site responsible primarily for the inhibitory action of choline esters was supposed to be a central site, distinct from the peripheral activity site proposed for the acceleration of decarbamoylation by pentanoylthiocholine [6].

Kinetic analysis of inhibition of decarbamoylation

In order to obtain more information on the association of the inhibitory choline esters with the central site of dimethylcarbamoyl-AChE, the inhibitory effect of laurylcholine on the decarbamoylation catalysed by choline, acting centrally, was examined kinetically. The plot of net first-order rate constants ($k_t - k'_t$) for choline-catalysed decarbamoylation against concentrations of choline showed a typical Michaelis-Menten type of kinetics, as had been observed previously [3,6], and in the presence of laurylcholine (0–100 μ M) the net decarbamoylation rate decreased markedly. When the net rate constants were subjected to double-reciprocal analysis (Figure 2a), laurylcholine appeared to inhibit choline-catalysed decarbamoylation competitively with a K_i value of 33 μ M, which is approx. 5 times lower than the IC_{50} value (170 μ M) in spontaneous decarbamoylation. Similar treatment (Figure 2b) of the data obtained for the inhibition by acetylcholine (0–20 mM) showed that choline-catalysed decarbamoylation was inhibited competitively by acetylcholine, with a K_i value of 7.6 mM. The affinity of acetylcholine decreased by 190-fold after modification of the active centre.

Figure 3 Inhibition of pentanoylthiocholine-induced decarbamylation by laurylcholine

Pentanoylthiocholine-induced decarbamylation of dimethylcarbamoyl-AChE was determined in the presence of laurylcholine as described in the Experimental section, and the net first-order constants ($k_t - k'_t$) in either the presence or the absence of laurylcholine were plotted against the concentrations (200–500 μM) of pentanoylthiocholine, where: k_t = observed first-order rate constant for pentanoylthiocholine-induced decarbamylation in the presence of laurylcholine (0–500 μM); k'_t = observed first-order rate constant for spontaneous decarbamylation in the presence of laurylcholine (0–500 μM).

Figure 4 Relationship between the inhibitory potency and size of the acyl group in choline esters

Acetylthiocholine-hydrolysing activity was measured in the presence of the choline esters at various concentrations as described in the Experimental section, and K_{IE} (○) and K_{ES} (●) values were determined by Lineweaver–Burk plot analysis. Spontaneous decarbamylation of dimethylcarbamoyl-AChE was carried out in the presence of the choline esters at various concentrations as described in Figure 1 legend, and IC_{50} (□) was expressed as the concentration giving 50% inhibition of spontaneous decarbamylation. The abscissa indicates the number of carbon atoms in the acyl chain of the choline ester.

In the following experiment, the inhibitory influence of laurylcholine on the decarbamylation induced by pentanoylthiocholine was examined. Since pentanoylthiocholine exerted an inhibitory effect at higher concentrations, a limited range of concentrations ($\leq 500 \mu\text{M}$) was used. As observed in Figure 3, the plot of net first-order rate constants ($k_t - k'_t$) for pentanoylthiocholine-induced decarbamylation against a concentration (200–500 μM) of pentanoylthiocholine showed a pattern characteristic of Michaelis–Menten kinetics (Figure 3). When laurylcholine (100 μM) was included, the net decarbamylation rate decreased by 45–50% at 400 or 500 μM pentanoylthiocholine.

Figure 5 Lineweaver–Burk plot for the inhibition of AChE activity by laurylcholine

The enzymic hydrolysis of acetylthiocholine (0.025–0.5 mM) was performed in the presence of laurylcholine (0–20 μM) as described previously [6] and the inhibition constants, K_{IE} (competitive) and K_{ES} (non-competitive), were obtained from Lineweaver–Burk plot analysis [1].

Figure 6 Effect of various choline esters on choline-catalysed decarbamylation of dimethylcarbamoyl- or monomethylcarbamoyl-AChE

Choline (1 mM)-catalysed decarbamylation of dimethylcarbamoyl-AChE (a) or monomethylcarbamoyl-AChE (b) was carried out in the presence of choline ester or thiocholine ester (1 mM) as described in Figure 1 legend. Abscissa indicates the number of carbon atoms in the acyl chain of the choline ester or thiocholine ester. (a) Choline ester (○); (b) choline ester (○) or thiocholine ester (●); 1 mM choline alone (-----); spontaneous decarbamylation (-----).

However, the increase in inhibitor concentration failed to result in the corresponding enhancement of the degree of inhibition, suggestive of no competition between pentanoylthiocholine and laurylcholine. Furthermore, the inhibitory action of laurylcholine appeared to be greater at higher concentrations of pentanoylthiocholine.

Association of choline esters with a long acyl chain with a hydrophobic zone

To show the ligand specificity in the binding of choline esters with a long acyl chain to the active site, the inhibitory effect of various choline esters (C_7 – C_{12}) on both enzymic hydrolysis of acetylthiocholine and spontaneous decarbamylation was analysed kinetically, and the kinetic constants, K_{IE} and K_{ES} , were compared with the IC_{50} value in the inhibition of spontaneous decarbamylation. Figure 4 shows that the inhibitory potency of each choline ester in both acetylthiocholine hydrolysis and spontaneous decarbamylation increased proportionally according to the length of the acyl chain (C_7 – C_{12}), with good

Figure 7 Effect of acetylcholine on spontaneous or choline-catalysed decarbamoylation of dimethylcarbamoyl- or monomethylcarbamoyl-AChE

Choline (1 mM)-catalysed decarbamoylation (●) of dimethylcarbamoyl-AChE (a) or monomethylcarbamoyl-AChE (b) was performed in the presence of acetylcholine as described in Figure 1 legend. Separately, the effect of acetylcholine on spontaneous decarbamoylation (○) was investigated by the method described in Figure 1(c): 1 mM choline alone (-----); spontaneous decarbamoylation (-----).

linearity, suggesting the involvement of the common hydrophobic domain in the respective inhibition. However, such a good relationship was not observed with choline esters containing acyl chains either shorter than the heptanoyl group or longer than the lauryl moiety. In related studies, the possible association of laurylcholine with the central site of acetyl-AChE was examined as described previously [1], where reversible inhibitors had been proposed to bind to a central site of acetyl-AChE, and produce the non-competitive component (K_{iES} value) in the inhibition of

acetylcholinesterase activity. Figure 5 demonstrates that laurylcholine inhibited the enzymic hydrolysis of acetylthiocholine with a mixed type of inhibition, where the K_{iE} and K_{iES} values were estimated to be 1.8 and 25 μ M, respectively. Interestingly, the non-competitive component (K_{iES} value) was found to be very close to the K_i value (33 μ M) of laurylcholine in choline-catalysed decarbamoylation.

Distinction between monomethylcarbamoyl- and dimethylcarbamoyl-AChE by using choline esters or thiocholine esters

Since it was likely that the steric effect could be exerted by alkyl substituents in the active centre of alkylcarbamoyl-AChE, an attempt was made to test this idea by using monomethylcarbamoyl-AChE and dimethylcarbamoyl-AChE, which have the same peripheral site, but a spatially different active centre. As illustrated in Figure 6, the potentiation of spontaneous decarbamoylation by choline (1 mM) and the dependence of the decarbamoylation rate on the size of the acyl group were commonly observed with these two modified enzymes. Generally, choline-catalysed decarbamoylation was higher for dimethylcarbamoyl-AChE than for monomethylcarbamoyl-AChE. Especially, there was a remarkable difference in the effect of ester compounds with acyl chains (C_2 – C_6) of a short or an intermediate size; whereas acetylcholine (1 mM) slightly enhanced choline-catalysed decarbamoylation of dimethylcarbamoyl-AChE, it decreased that of monomethylcarbamoyl-AChE below the control value (35% decarbamoylation). Although thiocholine esters with an acyl group of intermediate size (C_4 – C_6) were observed to accelerate choline-catalysed decarbamoylation of both modified enzymes, the overall decarbamoylation rate was smaller for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE. Additionally, the inhibition by choline esters with a long

Figure 8 (a) pH-dependence of decarbamoylation of dimethylcarbamoyl-AChE, (b) effect of ionic strength on the inhibition by laurylcholine and acetylcholine of the decarbamoylation of dimethylcarbamoyl-AChE

(a) Decarbamoylation was carried out at various pH values (6.0–8.0) in either the presence or the absence of each regulator. Half-life ($t_{1/2}$) was calculated from the first-order rate constant (k_1) as described previously [21]. Spontaneous decarbamoylation (○); pentanoylthiocholine (0.5 mM)-induced decarbamoylation (△); choline (0.5 mM)-catalysed decarbamoylation (●); choline (0.5 mM)-catalysed decarbamoylation in the presence of 0.5 mM pentanoylthiocholine (▲); 0.1 mM laurylcholine (■) or 2 mM acetylcholine (□). Inset, pH-dependence of choline-catalysed decarbamoylation. The rate constant ($k_1 - k_1'$) was plotted against pH (6.0–9.0). (b) Choline (1 mM)-catalysed decarbamoylation was determined in the presence of laurylcholine or acetylcholine as described in the Experimental section, except that the ionic strength of the buffer was varied: upper panels, 2 mM sodium phosphate buffer, pH 7.35; lower, the same buffer containing 150 mM NaCl with 0.5 mM choline alone (-----); spontaneous decarbamoylation (-----).

acyl group (C_7 – C_{12}) was greater for monomethylcarbamoyl-AChE than dimethylcarbamoyl-AChE. There was a similar relationship between the size (C_7 – C_{10}) of the acyl moiety and the inhibitory potency in both modified enzymes.

From these data, the decarbamylation rate in the system *in vivo* was expected to vary according to the concentration of intrinsic choline compounds or the alkyl group of the modified enzyme. In this respect, the concentration-dependent effect of acetylcholine on spontaneous or choline-catalysed decarbamylation was further investigated by using dimethylcarbamoyl-AChE and monomethylcarbamoyl-AChE. As exhibited in Figure 7, although total decarbamylation in the presence of 1 mM choline was similar for two modified enzymes, spontaneous decarbamylation differed greatly according to the alkyl group of the modified enzyme: approx. 4% for dimethylcarbamoyl-AChE and 10% for monomethylcarbamoyl-AChE. It is noteworthy that, whereas acetylcholine decreased spontaneous decarbamylation of dimethylcarbamoyl-AChE below control at more than 5 mM, the spontaneous decarbamylation of monomethylcarbamoyl-AChE was inhibited at a concentration as low as 1 mM. Meanwhile, total decarbamylation in the presence of 1 mM choline was slightly smaller for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE.

Effect of pH and ionic strength on decarbamylation

Figure 8(a) shows the pH-dependence of the decarbamylation rate over the range pH 6.0–8.0. The pH profile for decarbamylation of dimethylcarbamoyl-AChE was not altered significantly in the presence of either an accelerator or an inhibitor, somewhat consistent with the previous observation [21]. When the net first-order rate constant ($k_r - k_r'$) for choline-catalysed decarbamylation was plotted against pH, 50% of maximal decarbamylation was observed around pH 6.7 (Figure 8a, inset). Although a lower pK_a value of 5.5 had been suggested to be for a modulatory group on the peripheral site [22], it was not investigated, due to a lack of accuracy in this study. In a separate experiment, the effect of ionic strength on the inhibition of decarbamylation by laurylcholine or acetylcholine was investigated by using buffers of low or high ionic strength. Figure 8(b) demonstrates that the inhibitory action of either laurylcholine or excess acetylcholine was not remarkably affected by the change of ionic strength. Thus the role of the Coulombic force is not emphasized in the inhibition by laurylcholine or acetylcholine. Even the ionic-strength dependence of a lower-affinity phase was not altered significantly.

DISCUSSION

Acceleration of spontaneous decarbamylation by activatory choline esters is kinetically analogous to potentiation of choline-catalysed decarbamylation by these choline esters [6]; the maximal activation (approx. 2-fold) by pentanoyl- or hexanoylthiocholine, the same pH–decarbamylation profile, with a pK_a value of 6.7, a narrow range of AC_{50} values (50–70 μ M) and no dependence on ionic strength. Thus it is suggested that the potentiation effect of activatory choline esters occurs on the same peripheral site, which is designated tentatively as a peripheral activatory site, distinct from a peripheral inhibitory site for substrate inhibition [23].

In contrast with a strict structural requirement for the activatory action at the peripheral site [6], the inhibitory effect was expressed non-specifically by quaternary or tertiary ammonium compounds possessing various alkyl or acyl groups [6,11,24]. The involvement of different bindings sites in the

inhibition of spontaneous decarbamylation by choline esters is suggested from a wide range of IC_{50} values (0.35–25 mM). The inhibition by laurylcholine was assumed to be mainly due to the interaction of this compound with an active site rather than a peripheral site, since, in contrast with a negligible change in choline affinity [6], the binding affinity of laurylcholine decreased greatly after carbamylation of native enzyme. Moreover, the inhibition by laurylcholine was distinguished from the binding of peripheral aromatic ligands at a peripheral site [12] in the ionic-strength dependence. Especially, our findings that the inhibitory effect of choline esters with a long acyl group (C_7 – C_{12}) on both spontaneous decarbamylation and enzymic hydrolysis of acetylthiocholine increased proportionally according to the length of the acyl chain indicate the selective interaction of the acyl group (C_7 – C_{12}) with the hydrophobic domain in the central active site. The central binding seems to confer an additional binding affinity to the inhibitor ligand, as suggested from the relative affinity of laurylcholine (IC_{50} 170 μ M) and of acetylcholine (IC_{50} 10 mM) in spontaneous decarbamylation. These results are somewhat consonant with a previous report that the extension of the alkyl group beyond the octyl chain greatly enhanced the affinity of trimethylalkylammonium analogues at the active site of methylphosphono-AChE or native AChE [13]. From these data, it is proposed that the inhibition by laurylcholine in spontaneous decarbamylation involves both a quaternary ammonium-binding site and a hydrophobic domain in active site.

Laurylcholine binding to the active site is more positively suggested by the competitive inhibition of choline-catalysed decarbamylation by laurylcholine, although other interpretations could be possible. Furthermore, laurylcholine expressed a greater affinity for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE, posing a greater steric hindrance at the active centre. It is noteworthy that the binding affinity (K_i , 33 μ M) of laurylcholine in choline-catalysed decarbamylation was 5 times greater than that (IC_{50} , 170 μ M) in spontaneous decarbamylation, suggesting that the choline-binding site may be more contributory than the quaternary-ammonium-binding site in enhancing the ligand affinity. In addition, the selective association of acyl moiety (C_7 – C_{12}) with the active site is suggested from another result, that the inhibitory action of the choline ester on choline-catalysed decarbamylation of dimethylcarbamoyl-AChE increased in proportion to the length of the acyl group (Figure 6). From these data, it is suggested that the high-affinity binding of laurylcholine in the inhibition of choline-catalysed decarbamylation may involve both the choline-binding site and the hydrophobic domain.

From the data concerning the inhibition of pentanoylthiocholine-induced decarbamylation, it is unlikely that laurylcholine interferes with pentanoylthiocholine-induced decarbamylation through binding to the peripheral activity site for pentanoylthiocholine. Instead, although there could be other interpretations of inhibition kinetics, a central binding is suggested for the inhibition by laurylcholine; the concentration of laurylcholine for 50% inhibition of pentanoylthiocholine-induced decarbamylation was closer to the higher binding affinity (IC_{50} , 170 μ M) of laurylcholine in spontaneous decarbamylation. Moreover, the pattern for the inhibition by laurylcholine in pentanoylthiocholine-catalysed decarbamylation was similar to that in the spontaneous decarbamylation. Thus it seems that the active site of carbamoyl-AChE is recognized similarly by laurylcholine in either pentanoylthiocholine-induced or spontaneous decarbamylation.

Acetylcholine binding to the active centre of dimethylcarbamoyl-AChE is derived from the results that acetylcholine competitively (K_i 7.6 mM) inhibited choline-catalysed de-

Figure 9 Proposed model for multiple interactions of various choline esters with dimethylcarbamoyl-AChE

I, choline esters with short acyl chain (C_2 – C_3); II, choline esters with acyl chain of intermediate size (C_4 – C_6); III, choline esters with long acyl chain (C_7 – C_{12}).

carbamoylation of dimethylcarbamoyl-AChE, and that its inhibitory effect in either spontaneous or choline-catalysed decarbamoylation was greater for monomethylcarbamoyl-AChE than for dimethylcarbamoyl-AChE (Figure 6). It is not surprising to find that the affinity difference (190-fold) of acetylcholine between the native enzyme and dimethylcarbamoyl-AChE was comparable with that (130-fold) of edrophonium, another active-centre-specific ligand, between native enzyme and monomethylcarbamoyl-AChE [13]. Compared with the 18-fold decrease in laurylcholine affinity after modification of the active site, a 190-fold decrease in acetylcholine affinity, which demonstrates the loss of an acetyl-moiety contribution to the complex stabilization in the active centre, may be compatible with the suggestion [25] that the acetyl-binding subsite in the active centre is not on the same loci as the acyl-binding region in the hydrophobic domain.

On the other hand, a lower inhibitory action ($K_i > 15$ mM) of choline esters with an acyl chain of intermediate size (C_4 – C_6), compared with acetylcholine, may be explained by the notion that the entry of these compounds to the active centre may be sterically hindered. In support of this idea, the inhibitory action of these choline esters was smaller for dimethylcarbamoyl-AChE than for monomethylcarbamoyl-AChE, and that thiocholine esters with a more-expanded structure were less inhibitory than choline esters in both spontaneous and choline-catalysed decarbamoylation. In addition, these choline esters may be inappropriate for the association with the hydrophobic region or away from it, as suggested from the greater difference in inhibitory potency between hexanoylcholine (IC_{50} 23.1 mM) and heptanoylcholine (IC_{50} 7.7 mM), in comparison with the 1.5-fold difference between IC_{50} values of heptanoylcholine and octanoylcholine. Therefore, both the steric exclusion from the active centre and the poor interaction with the hydrophobic site might be the reasons why the esters with an acyl moiety of an intermediate size are less inhibitory. This might explain why thiocholine esters, less inhibitory in substrate hydrolysis, were more efficient than choline esters in accelerating the decarbamoylation, and that pentanoylthiocholine and hexanoylthiocholine, with higher K_i values [6], were more effective in enhancing the decarbamoylation of

dimethylcarbamoyl-AChE or monomethylcarbamoyl-AChE. Thus the action, inhibitory or activatory, of choline esters is determined by the relative difference in binding affinity of ligands for the active site relative to the peripheral activatory site.

Binding of choline esters with a long acyl group (C_7 – C_{12}) to the active site could also occur in acetyl-AChE. The parallel relationship between the K_i value and the K_{iES} value of the respective choline ester in the hydrolysis of acetylthiocholine suggests that the non-competitive component (K_{iES} value) is likely to be due to the binding of choline esters to the active site. This might be further supported by the similarity between the K_i value of laurylcholine in choline-catalysed decarbamoylation and the K_{iES} value in substrate hydrolysis. Thus it is proposed that choline esters may interact with acetyl-AChE as well as carbamoyl-AChE in an identical mode.

On the basis of all these data, the interaction of choline esters with AChE can be classified into three types of binding, according to the size of the acyl chain as shown in Figure 9. These results would be useful for the understanding of various possible binding subsites characterized by differences in charge effect, steric constraint and/or hydrophobicity. Also, this study would be informative in characterizing the properties and ligand specificity of binding sites in cholinesterase from various sources.

This work was supported in part by the Agency for Defense Development.

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Received 17 September 1993/17 March 1994; accepted 23 March 1994